Polyploidization-induced genome variation in triticale

Xue-Feng Ma, Peng Fang, and J. Perry Gustafson

Abstract: Polyploidization-induced genome variation in triticale (× *Triticosecale* Wittmack) was investigated using both AFLP and RFLP analyses. The AFLP analyses were implemented with both *Eco*RI–*Mse*I (E–M) and *Pst*I–*Mse*I (P–M) primer combinations, which, because of their relative differences in sensitivity to cytosine methylation, primarily amplify repetitive and low-copy sequences, respectively. The results showed that the genomic sequences in triticale involved a great degree of variation including both repetitive and low-copy sequences. The frequency of losing parental bands was much higher than the frequency of gaining novel bands, suggesting that sequence elimination might be a major force causing genome variation in triticale. In all cases, variation in E–M primer-amplified parental bands was more frequent in triticale than that using P–M primers, suggesting that repetitive sequences were more involved in variation than low-copy sequences. The data also showed that the wheat (*Triticum* spp.) genomes were relatively highly conserved in triticales, especially in octoploid triticales, whereas the rye (*Secale cereale* L.) genome consistently demonstrated a very high level of genomic sequence variation (68%–72%) regardless of the triticale ploidy levels or primers used. In addition, when a parental AFLP band was present in both wheat and rye, the tendency of the AFLP band to be present in triticale was much higher than when it was present in only one of the progenitors. Furthermore, the cDNA-probed RFLP analyses showed that over 97% of the wheat coding sequences were maintained in triticale, whereas only about 61.6% of the rye coding sequences were maintained, suggesting that the rye genome variation in triticale also involved a high degree of rye coding sequence changes. The data also suggested that concerted evolution might occur in the genomic sequences of triticale. In addition, the observed genome variation in wheat–rye addition lines was similar to that in triticale, suggesting that wheat–rye addition lines can be used to thoroughly study the genome evolution of polyploid triticale.

Key words: wheat, rye, polyploid, genome evolution, sequence elimination.

Résumé : Les changements génomiques induits par la polyploïdisation chez le triticale (× *Triticosecale* Wittmack) ont été examinés à l'aide d'analyses AFLP et RFLP. Les analyses AFLP ont été réalisées tant avec les amorces *Eco*RI–*Mse*I (E–M) que *Pst*I–*Mse*I (P–M), lesquelles amplifient principalement des séquences répétées ou à faible nombre de copies, respectivement, en raison de leur sensibilité différente à la méthylation des cytosines. Les résultats révèlent que les séquences génomiques du triticale montrent une grande variation tant chez les séquences répétées que chez celles à faible nombre de copies. La disparition d'une bande parentale était beaucoup plus fréquente que l'apparition de bandes nouvelles ce qui suggère que l'élimination de séquences serait une force majeure contribuant à la variation génomique chez le triticale. Dans tous les cas, la variation au niveau des bandes produites avec les amorces E–M était plus fréquente que celle révélée au moyen des amorces P–M. Cela suggère que les séquences répétées sont plus impliquées dans la variation que ne le sont les séquences à faible nombre de copies. Les données ont également révélé que les génomes du blé (*Triticum* ssp.) étaient relativement très bien conservés chez les triticales, particulièrement chez les triticales octoploïdes, tandis que le génome du seigle (*Secale cereale* L.) montrait régulièrement une très grande variabilité génomique (68 % à 72 %) sans égard au niveau de ploïdie du triticale ou aux amorces employées. Des plus, quand une bande AFLP parentale était présente tant chez le blé que chez le seigle, la probabilité de trouver cette bande chez le triticale était beaucoup plus élevée que lorsque la bande ne provenait que d'un seul des deux parents. Finalement, les analyses RFLP, réalisées à l'aide de sondes d'ADNc, ont montré que 97 % des régions codantes du blé étaient présentes chez le triticale tandis que seulement 61,6 % des séquences codantes du seigle étaient conservées. Ces résultats suggèrent que la variation du génome du seigle au sein du triticale comprend beaucoup de changements au niveau des séquences codantes du seigle. Ils suggèrent également que de l'évolution concertée pourrait avoir lieu dans

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les séquences génomiques du triticale. Enfin, la variation génomique observée au sein de lignées d'addition blé–seigle est semblable à celle observée chez le triticale, une observation qui suggère que des lignées d'addition blé–seigle pourraient s'avérer utiles pour étudier en détail l'évolution des génomes chez le triticale polyploïde.

Mots clés : blé, seigle, polyploïde, évolution du génome, élimination de séquences.

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Introduction

Polyploidy is a prominent process of speciation in the plant kingdom. It has been estimated that at least 50%, and perhaps more than 70%, of angiosperms have experienced polyploidy one or more times at some point in their evolutionary history (Masterson 1994; Wendel 2000). Because of its widespread coverage and great importance in crops, polyploidy, most importantly allopolyploidy, has received considerable attention and research interest during the last 50 years (Soltis and Soltis 1995). However, little was known about the genome evolution of polyploids until recent molecular studies. Song et al. (1995) stimulated the research interest in polyploid genome evolution by studying newly synthesized tetraploids of *Brassica*. Newly synthesized polyploids provide opportunities to investigate early genetic and epigenetic events that accompany polyploid formation. This strategy has been widely used in many species, including *Arabidopsis* (Comai et al. 2000; Madlung et al. 2002), *Brassica* (Song 1995), cotton (*Gossypium* spp.) (Liu et al. 2001), and wheat (*Triticum* spp.) and its wild relatives (*Aegilops* spp.) (Ozkan et al. 2002; Feldman and Levy 2003). However, only wheat has been used for a series of molecular studies, which have provided considerable novel insight into the evolution of polyploids (Feldman et al. 1997; Liu et al. 1998*a*, 1998*b*; Shaked et al. 2001; Ozkan et al. 2001, 2002, 2003; Kashkush et al. 2002, 2003; Feldman and Levy 2003).

The overall results from wheat indicated that rapid sequence elimination in synthetic allopolyploids was a common, nonrandom, directional, highly reproducible event whose direction was determined by the genomic combination of the amphiploids, although some of the changes occurred in a random fashion. Sequence elimination was not affected by the parental genotype, cytoplasm, or ploidy level; and it did not result from intergenomic recombination. It has been speculated that specific sequence elimination augmented the differentiation of homoeologous chromosomes at the polyploid level, thus providing the physical basis for the diploid-like meiotic behavior characterizing polyploid wheat. Accordingly, rapid elimination of these sequences improved the fitness of newly formed allopolyploids, thus facilitating their rapid establishment in nature as a successful new species (Ozkan et al. 2002; Feldman and Levy 2003). In addition, polyploidization-induced rapid epigenetic modifications have been reported in wheat (Kashkush et al. 2002, 2003). Similar genetic changes and (or) epigenetic variations have also been observed in *Arabidopsis* (Comai et al. 2000; Lee and Chen 2001; Madlung et al. 2002), *Brassica* (Song et al. 1995), and triticale (× *Triticosecale* Wittmack) (Ma et al. 2002). However, in complete contrast, the recent amplified fragment length polymorphism (AFLP) and methylation-sensitive amplification polymorphism (MSAP) analyses in cotton indicated quiescent genome behavior, where parental fragments were perfectly conserved in the tetraploids without sequence elimination or epigenetic modifications (Liu et al. 2001). A similar phenomenon has also been reported in the young allopolyploid species *Spartina anglica* (Baumel et al. 2001, 2002, 2003), which demonstrated strict additivity of parental subgenomes in the resulting allopolyploids. But a quiescent coexistence of parental genomes does not entail that all genes from both parents are coexpressed.

Adams et al. (2003) assayed homoeologous gene contributions in both natural and synthetic tetraploid cotton by cDNA single-stranded conformation polymorphism (cDNA-SSCP) analysis. The results revealed a 25% transcription bias among the 40 homoeologous gene pairs studied, with altered gene expression induced either by gene silencing or by unequal expression of the duplicated genes. It also suggested that similar homoeologue expression and silencing patterns were sometimes displayed between natural and synthetic tetraploids, suggesting both immediate and long-term response to polyploidization (Adams et al. 2003). The overall data in newly formed cotton tetraploids indicated immediate epigenetic modifications rather than immediate genetic changes.

The contradictory results among cotton, *S. anglica*, and other species indicate that different species may have different approaches to achieve stabilized diploid-like allopolyploids. Because of rapid progress in this field, the phenomena and potential mechanisms associated with polyploid evolution have been recently reviewed from various aspects (Galitski et al. 1999; Comai 2000; Soltis and Soltis 2000; Wendel 2000; Eckardt 2001; Rieseberg 2001; Wolfe 2001; Liu and Wendel 2002; Ozkan et al. 2002; Feldman and Levy 2003; Osborn et al. 2003; Wendel and Cronn 2003; Ma and Gustafson 2004). Although these studies greatly extended our knowledge regarding the genome evolution of polyploids, the genetic mechanisms contributing to polyploid genome evolution remain elusive. These studies mainly focused on a few species. An extensive study of the topic needs to consider more materials from other species. Among those to be studied, the man-made species triticale is one of the best candidates.

Triticale, the first synthesized amphiploid cereal, is a hybrid between wheat and rye (*Secale cereale* L.). Since the first artificial hybridization between wheat and rye (Wilson 1876), thousands of primary triticale lines with various ploidy levels and genome combinations, such as AARR, AABBRR, and AABBDDRR, have been created. Furthermore, secondary triticale lines, which are the stable hexaploid derivatives obtained by intercrossing an octoploid triticale or a hexaploid wheat with a hexaploid triticale, have been developed for commercial production. Because of its short history, the complete pedigree of each triticale is

known, which provides a series of model materials for the investigation of genome evolution following polyploid formation (Ma et al. 2002; Voylokov and Tikhenko 2002).

Similar to other polyploid species, the genome of triticale has undergone post-polyploidization alteration. Phenotypic instability has been noticed in primary triticales (Voylokov and Tikhenko 2002). Cytological observations have shown that the morphology of rye chromosomes is modified when placed into a wheat background (Gustafson 1976; Alkhimova et al. 1999). Furthermore, many researchers (Lacadena et al. 1984; Somers and Gustafson 1994; Gustafson and Flavell 1996; Viegas et al. 1996; Houchins et al. 1997; Neves et al. 1997; Rozynek et al. 1998) have reported the changes of rye gene expression in triticale.

The primary molecular studies implemented by Ma et al. (2002) indicated that most of the repetitive sequences present in the wheat parent were maintained in triticale, but more than 70% of the repetitive sequences present in rye were lost or altered in triticale. The results also showed that hexaploid wheat genomes were more conserved in octoploid triticale when compared with tetraploid wheat genomes in hexaploid triticale (Ma et al. 2002). Another study demonstrated that there was a considerable degree of DNA content decrease in the course of triticale formation with about a 9% decrease for octoploid triticale and a 28%–30% decrease for hexaploid triticale (Boyko et al. 1984). The data suggested that considerable repetitive sequence alteration or loss occurred in triticale after its formation. However, little is known about the changes regarding low-copy coding sequences.

The present study investigated the overall genome variation occurring within both low-copy and repetitive sequences using a large set of AFLP primers, including both methylation-sensitive and -insensitive enzymes. AFLP analysis provided an opportunity to investigate genomic sequence changes at a genome-wide level. However, exactly how representative of the entire genome amplified sequences are depends on the restriction enzymes used in the study. A general case affecting restriction enzyme cleavage is cytosine methylation, which is widespread in the genome and predominately present in CpG or CpNpG sites (Gruenbaum et al. 1981) and in non-coding and repetitive sequences (Finnegan et al. 1998). Coding sequences are usually not methylated. Thus, an unbiased investigation should use enzyme combinations with the ability to cleave both coding, low-copy sequences, and non-coding, repetitive sequences. Therefore, two kinds of primer combinations, *Eco*RI–*Mse*I (E–M) and *Pst*I–*Mse*I (P–M), were used in this study. The cleavage of *Eco*RI is usually not affected by the cytosine status in CpG or CpNpG sites, although it can be inhibited by cytosine methylation in its own recognition site, whereas *Pst*I is highly sensitive to cytosine status in CpNpG sites because its recognition sites involves both CTG and CAG trinucleotides. As a result, *Pst*I predominantly cleaves low-copy sequences, whereas *Eco*RI mainly cleaves repetitive sequences. Thus, the AFLP marker distributions of the two types of markers are different. In wheat, E–M markers are evenly distributed along a chromosome, whereas most P–M markers are present in the distal region of a chromosome arm owing to high heterochromatin content near centromere (Rodriguez Milla and Gustafson 2001). Similar results were also reported in soybean (*Glycine max* L.) (Young et al. 1999) and maize (*Zea mays* L.) (Vuylsteke et al. 1999). The current study provides an overall estimation of genomic sequence variation using both E–M and P–M primers.

Over 13 000 AFLP bands were scored from hexaploid and octoploid triticales, as well as from wheat–rye addition lines. Because of the genome-wide property of AFLP analyses, the large set of data should provide an unbiased estimation of the overall genome variation occurring during and after the formation of triticale. Furthermore, cDNA-probed RFLP analyses were also performed in this study, which should give a better estimation of the genetic variation of expressed sequences.

Materials and Methods

Plant materials and DNA isolation

Four primary triticales, including *Triticum turgidum* 'Cocorit 71' × *Secale cereale* 'Snoopy' (2*n* = 6x, AABBRR), 'Cocorit 71' × *Secale cereale* 'UC90' (2*n* = 6*x*, AABBRR), *Triticum aestivum* 'Chinese Spring' × *Secale cereale* 'Imperial' (*2n* = 8*x*, AABBDDRR), and *Triticum aestivum* 'Holdfast' × *Secale cereale* 'King II' (2*n* = 8*x*, AABBDDRR), two sets of wheat – rye addition lines, which correspond to the two octoploid triticales, and their wheat and rye progenitors were used in this study. The genome of each wheat–rye addition line is composed of a single pair of rye homologous chromosomes plus an entire wheat genome. The two octoploid triticales and their corresponding wheat–rye addition lines are at least 35 generations old, whereas the two hexaploid triticales are about 15 generations old after their formation. All seed stocks were obtained from the USDA–Sears collection, Columbia, Mo. Genomic DNA was isolated from dried young leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Saghai Maroof et al. 1984).

AFLP analysis

The original protocol of Vos et al. (1995) was followed with some modifications. Digestion of genomic DNA (500 ng) with *Eco*RI or *Pst*I and *Mse*I restriction enzymes and ligation were performed separately in 1 × One-Phor-All buffer (Amersham Pharmacia Biotech, Piscataway, N.J.) in a final volume of 60 μL. For preamplification, 2.5 μL of the digestion–ligation reaction were used in a 20-μL reaction volume containing primers (75 ng) with one selective nucleotide, 0.2 mM of each dNTP, 1 U *Taq* polymerase, and 1× *Taq* polymerase buffer containing 1.5 mM $MgCl_2$. *Eco*RI or *Pst*I primers were 5′ end labeled with [γ-^{33}P]ATP using 0.1 U of T4 PNK (polynucleotide kinase) and 1× One-Phor-All buffer. The preamplification reaction was diluted 20 fold and 2.5 μL were used for a second amplification step with primers containing three selective nucleotides in a 10-μL reaction containing 2.5 ng of the labeled *Eco*RI or *Pst*I primer, 15 ng of *Mse*I primer, 0.2 mM of each dNTP, 0.25 U *Taq* polymerase, and 1× *Taq* polymerase buffer containing 1.5 mM $MgCl_2$. All reactions were performed in a PCR Express thermal cycler (Hybaid, Franklin, Mass.) using 96-well plates. Marker designations contained the primer combination used (abbreviated by the initial of the restriction enzyme, followed by the selective nucleotides). Forty AFLP primer combinations were used for triticale analysis, and eight of

them were used for addition lines. AFLP banding profiles were scored as presence or absence for each group of materials of wheat, rye, triticale, and addition lines.

RFLP analysis

The nuclear size in terms of 2C DNA content for triticale is estimated as the sum of wheat and rye. Since 2C DNA contents are very different for wheat (26.3 pg for 4*x* and 36.2 pg for 6*x*), rye (18.9 pg), and triticale (45.2 pg for 6*x* and 55.1 pg for 8*x*), the amounts of DNA digested for each group of materials of wheat, rye, and triticale were adjusted based on their ratio of 2C nuclear DNA contents (Bennett and Leitch 1995). DNA samples were digested with each of the five restriction enzymes, including *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, and *Hin*dIII, with a final concentration of 2.5 U per μg DNA, electrophoresed on 0.7% *w*/*v* agarose gels at 30 V for 16–18 h, and alkali transferred to Hybond-N+ membranes (Amersham Pharmacia Biotech) following the manufacturer's instructions. Fifty barley (*Hordeum vulgare* L.) or oat (*Avena sativa* L.) cDNA clones (Heun et al. 1991; Van Deynze et al. 1998) were used as probes for Southern analysis. Probes were prepared by PCR amplification of inserts and 50–100 ng were labeled with [α-^{32}P]dCTP using the High Prime Kit (Roche, Indianapolis, Ind.). Hybridization was performed in a solution containing 5× SSC, 5× Denhardts, 0.5% *w*/*v* SDS, and 100 μg denatured salmon sperm DNA/mL at 65 °C for 12–16 h. After hybridization, membranes were washed with a final stringency of 0.1× SSC and 0.1% *w*/*v* SDS for 30 min at 65 °C and exposed to X-ray film at –80 °C for 2–6 days.

Results

AFLP analysis in triticale

The overall genomic sequence variation was evaluated based on AFLP banding profiles (Fig. 1) in triticales. For each primer combination, AFLP bands were scored as present or absent for each group of materials including wheat, rye, and their corresponding triticale. In total, more than 6800 bands were scored for each type of primer combination and classified into seven kinds of banding patterns (Table 1). The proportions of each banding pattern were the same among triticale ploidy levels (hexaploid vs. octploid) within the same enzyme group, but they were very different between the two types of primers (E–M vs. P–M).

The data were further grouped into four major classes based on banding presence or absence of the parental wheat and rye patterns (Table 1). The total number of bands was calculated for each major class, revealing that the proportions of wheat parental bands (class + –) were roughly at the same level for both E–M (47.5%) and P–M (49.4%) primers, whereas the proportions of the other three classes displayed considerable differences between two types of primers.

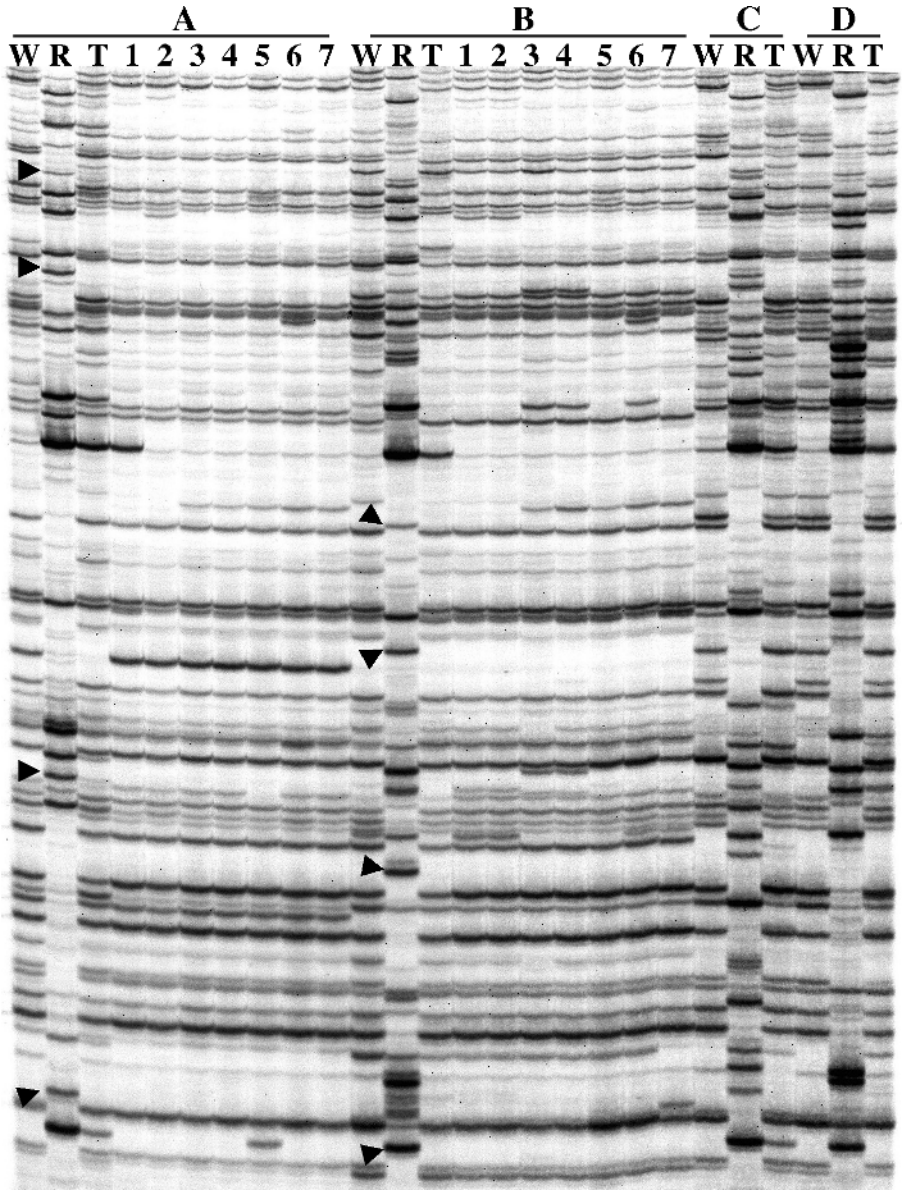
The data were also evaluated to determine how many parental bands showed Mendelian (additivity) inheritance (Table 2). Of all the materials studied, P–M primers consistently showed much higher additive inheritance (on average, 61.0%) than E–M primers (on average, 48.7%). As expected, this indicated that low-copy sequences were more conserved in triticale than repetitive sequences. In addition, octoploid triticales had a higher percentage of additive inheritance than hexaploid triticales for both types of primers, especially for E–M primers, where there was more than a 10% difference in the percentage of Mendelian inheritance between the two ploidy levels. This suggested that genomic sequences in hexaploid triticale underwent greater changes than those in octoploid triticale, but that the degree of sequence variation was low within low-copy sequences (P–M primers); the degree of sequence variation was very high within repetitive sequences (E–M primers).

The types of sequence variation were further investigated based on the changing events of each banding pattern. Among the seven kinds of banding patterns, six were polymorphic, and four involved the loss of parental bands or the appearance of novel bands (Table 3). The relatively higher conservation of parental AFLP bands in P–M primers than in E–M primers was due to the smaller percentages of lost and novel bands (33.2% and 5.8%, respectively) when using P–M primers than when using E–M primers (41.6% and 9.7%, respectively). The data also indicated that the consistency of sequence variation among different triticales using P–M primers was due to a similar proportion of parental band disappearance or novel band appearance. In contrast, when E–M primers were used, the frequencies of band loss were very different (χ^2 value is the highest) between hexaploid (on average, 48.9%) and octoploid (on average, 34.6%) triticales. In all cases, the frequencies of lost parental bands were much higher than the frequencies of gained novel bands, suggesting that sequence loss, which occurred mainly among repetitive sequences, might be a major factor causing genome variation in triticale.

Genomic sequence loss was further characterized based on wheat or rye parents (Table 1). Those bands showing the same patterns for wheat and rye, but differing in triticale, were used to calculate the proportions of presence and absence of AFLP bands in the resulting triticale. Overall, 38.7% (E–M primers) and 13.4% (P–M primers) of the wheat parental bands were lost, whereas 68.0% (E–M primers) and 72.5% (P–M primers) of the bands present in rye disappeared, again suggesting that the rye genome underwent significantly greater changes than the wheat genome when present in a triticale background. Comparison of AFLP bands between octoploid and hexaploid triticales showed that the proportions of presence and absence of wheat parental bands were very similar between the two octoploid and the two hexaploid triticales; however, as previously stated, there was a significant difference between different polyploidy levels of triticales, as well as between two kinds of primers.

The ploidy difference in sequence variation was further calculated for each kind of primer based on the data in Table 1. The ratio of presence vs. absence of wheat AFLP bands was approximately 2.7:1 for E–M primers and 9.0:1 for P–M primers in octoploid triticales, but only about 1:1 (E–M primers) and 4.8:1 (P–M primers) in hexaploid triticales, respectively. This result indicated that hexaploid wheat genomes were more conserved in octoploid triticale when compared with tetraploid wheat genomes in hexaploid triticale. However, for the rye genome, the ratios of presence vs. absence of rye AFLP bands in hexaploid triticales were roughly the same as in octoploid triticales, and they were also very similar between the two types of primers (from 1:2

Fig. 1. AFLP banding profiles of the four triticales and two sets of addition lines as amplified by PACA–MCTT. (A) 'Chinese Spring' × Imperial. (B) Holdfast × King II. (C) Cocorit 71 × Snoopy. (D) Cocorit 71 × UC90. W, wheat; R, rye; T, triticale. The numbers from 1 to 7 represent seven wheat–rye addition lines from 1R to 7R of the corresponding wheat and rye materials. Arrowheads indicate some of those rye bands that are absent in triticale and addition lines.



to 1:3). These results suggested that the wheat parent ploidy level of triticale played an important role in determining wheat AFLP fragment variation levels, whereas rye genomic sequences consistently showed a considerable degree of variation (on average, 70.3%) regardless of the primer types or the wheat parental ploidy levels.

The results also showed that when a parental AFLP band was present in both wheat and rye (class + +, Table 1), the tendency of that AFLP band to be present in triticale was much higher than when it was present in only one of the progenitors. This tendency can be seen in all cases regardless of the primer types or materials used (Table 1). Because of the high degree of sequence conservation in octoploid triticales, when P–M primers were used, the resulting data indicated that low-copy sequences were significantly preserved in octploid triticale (over 92%) when they were present in both of its progenitors.

Some of these bands (+ + +) may be derived from the effect of combining two kinds of banding patterns (+ – + and – + +, Table 1). If this happens, the probabilities of type + + + are estimated as 2.6% (2008/6894 × 614/6894) for E–M primers and 4.2% (2942/6872 × 670/6872) for P–M primers. However, the observed percentages of type + + + were 10.7% (736/6894) for E–M primers and 8.4% (577/6872) for P–M primers. This indicated that when a parental AFLP band was present in both parents of a triticale, the tendency of that band to be present in triticale was, on average, 4.1 times, for E–M primers, and 2 times, for P–M primers, the chance of any band that was present in only one of the progenitors. The data clearly showed the correlation

Table 1. Numbers of AFLP fragments of each banding pattern and the percentages of presence or absence of parental bands.

				CS×I		H×K		C×S		C×U		Total	
Primers	W	R	T	No.	%	No.	%	No.	%	No.	%	No.	%
*Eco*RI–*MseI*	+	–	+	629	74	568	72	419	52	392	47	2008	61
			–	223	26	221	28	386	48	440	53	1270	39
	–	+	+	220	36	147	32	155	32	92	25	614	32
			–	388	64	311	68	328	68	278	75	1305	68
	+	+	+	231	83	166	88	189	66	150	55	736	72
			–	47	17	23	12	99	34	124	45	293	29
	–	–	+	172		164		160		172		668	
	Total			1910		1600		1736		1648		6894	
*Pst*I–*Mse*I	+	–	+	821	89	780	91	690	84	651	82	2942	87
			–	99	11	78	9	136	17	142	18	455	13
	–	+	+	146	25	158	26	216	33	150	26	670	28
			–	449	76	460	74	435	67	421	74	1765	73
	+	+	+	151	93	151	91	161	90	114	85	577	90
			–	11	7	15	9	18	10	20	15	64	10
	–	–	+	91		86		120		102		399	
	Total			1768		1728		1776		1600		6872	

Note: CS×I, 'Chinese Spring' × 'Imperial'; H×K, 'Holdfast' × 'King II'; C×S, 'Cocorit 71' × 'Snoopy'; C×U, 'Cocorit 71' × 'UC90'; W, wheat; R, rye; T, triticale; +, present; –, absent.

Table 2. AFLP fragment additivity in triticales.

		CS×I		H×K		C×S		C×U		Total	
Primers	Banding Type	No.	%	No.	%	No.	%	No.	%	No.	%
*Eco*RI–*Mse*I	Additive	1080	57	881	55	763	44	634	39	3358	49
	Non-additive	830	44	719	45	973	56	1014	62	3536	51
*Pst*I–*Mse*I	Additive	1118	63	1089	63	1067	60	915	57	4189	61
	Non-additive	650	37	639	37	709	40	685	43	2683	39

Note: CS×I, 'Chinese Spring' × 'Imperial'; H×K, 'Holdfast' × 'King II'; C×S, 'Cocorit 71' × 'Snoopy'; C×U, 'Cocorit 71' × 'UC90'.

Table 3. Numbers and percentages of AFLP fragments changed in triticales.

Primers	No. of Bands	CS×I	H×K	C×S	C×U	Total	χ^2 value
*Eco*RI–*Mse*I	Total	1910	1600	1736	1648	6894	
	Polymorphic (%)	1679 (87.9)	1434 (89.6)	1547 (89.1)	1498 (90.9)	6158 (89.3)	0.52
	Loss (%)	658 (34.5)	555 (34.7)	813 (46.8)	842 (51.1)	2868 (41.6)	5.15
	Novel (%)	172 (9.0)	164 (10.3)	160 (9.2)	172 (10.4)	668 (9.7)	0.16
*Pst*I–*Mse*I	Total	1768	1728	1776	1600	6872	
	Polymorphic (%)	1617 (91.5)	1577 (91.3)	1615 (90.9)	1486 (92.9)	6295 (91.6)	0.02
	Loss (%)	559 (31.6)	553 (32.0)	589 (33.2)	583 (36.4)	2284 (33.2)	0.43
	Novel (%)	91 (5.1)	86 (5.0)	120 (6.8)	102 (6.4)	399 (5.8)	0.43

Note: CS×I, 'Chinese Spring' × 'Imperial'; H×K, 'Holdfast' × 'King II'; C×S, 'Cocorit 71' × 'Snoopy'; C×U, 'Cocorit 71' × 'UC90'.

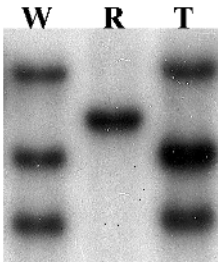
between the similarity and preservation of sequences, especially for repetitive sequences in triticale. The low-copy sequences showed a lower degree of conservation in this comparison because P–M primers predominantly amplified coding sequences that were usually hyper-methylated and thus blocked from cleavage by *Pst*I when multiple copies existed. It is obvious that parental sequence similarity has played a significant role in genome introgression and variation during intergeneric hybridization and allopolyploidization in triticale.

The overall data revealed by AFLP analysis suggested that low-copy sequences had a higher tendency to be conserved in triticale than repetitive sequences, and this tendency was even more pronounced in octoploid triticale than in hexaploid triticale. Because most coding sequences were low-copy, the results implied that the conservation of functionally expressed coding sequences was much higher than that of non-coding sequences. However, since the amplified AFLP fragments were anonymous, it was impossible to absolutely distinguish coding from non-coding sequences using the genomic AFLP analyses. Thus, in this study, cDNA-probed RFLP analyses were also performed to investigate the behavior of expressed sequences during the course of triticale formation.

RFLP analysis in triticale

The same set of triticale materials was used for Southern hybridization (Fig. 2) with five restriction enzymes. Fifty

Fig. 2. RFLP banding pattern of CDO595 in 'Chinese Spring' × 'Imperial' when it was digested with *Eco*RV. W, wheat; R, rye; T, triticale. The figure shows the missing rye parental band in the triticale.



barley or oat cDNA clones were selected as probes based on their distribution on the rye chromosomes (Ma et al. 2001; Van Deynze et al. 1998). The resulting data were organized in the same manner as AFLP analyses (Table 4). An interesting observation, which differed from the AFLP analyses, was that different materials demonstrated a similar percentage of bands for each banding pattern, regardless of ploidy level. However, the data were in agreement with the AFLP analyses in that sequence changes were also very common and highly directed. The data also showed that sequence loss contributed to most of the variation, since the number of bands lost (691) was much higher than the number of new bands (250). Sequence elimination could be visualized from Fig. 2, where the corresponding rye parental band was not present in triticale. However, the intensity of the middle band in triticale was much higher than that of the corresponding wheat band, suggesting that the size of the rye parental band was decreased to a size that was equal to the size of the middle band in wheat parent. Because the restriction enzyme used was *Eco*RV, which is not sensitive to the status of cytosine methylation, the observed banding size decrease should be caused by sequence elimination. In addition, the observed rye banding decrease in Fig. 2 might imply concerted evolution (Elder and Turner 1995; Wendel et al. 1995; Cronn et al. 1996), because the rye parental band has been homogenized to one of the wheat parental bands.

Similar to the AFLP data, the RFLP results showed that low-copy coding sequences were highly conserved in triticale. However, the RFLP data displayed an even larger degree of sequence maintenance than the AFLP data for both parents owing to the fact that the cDNA clones were all derived from coding sequences, whereas AFLP fragments contained a variety of undefined sequences. Noticeably, wheat RFLP bands were remarkably preserved in all triticales. Over 97% of the wheat RFLP bands were preserved in triticales, whereas 86.6% were preserved when AFLP (P–M primers, Table 1) analyses were performed. This suggested that over 10% of the low-copy AFLP fragments derived from wheat were non-coding sequences. The rye cDNA bands showed a similar tendency toward increased conservation compared with that observed in AFLP analyses, but the overall degree of conservation was still very low (38.4%). The data reinforced the AFLP findings that rye parental genomic sequences underwent a great degree of change, which included 61.6% of the rye coding sequences, in a triticale background. The results also indicated a very high degree (97.5%) of banding conservation when parental fragment sizes were the same between wheat and rye. In addition, the RFLP data revealed the appearance of about 7.3% (250/3416) new bands, which in most cases were correlated with the disappearance of rye parental bands, indicating that some of the banding loss was caused by DNA methylation or sequence changes.

AFLP analysis in wheat–rye addition lines

A unique advantage of triticale is that there exists two sets of wheat–rye addition lines, which correspond to two octoploid triticales, 'Chinese Spring' × 'Imperial' and 'Holdfast' × 'King II'. Each addition line contains a single pair of rye chromosomes in hexaploid wheat. These addition lines were derived from backcrossing octoploid triticales with their wheat parents. Thus, studying the genome variation of addition lines might provide interesting insight into the genome evolution of triticale. In addition, addition lines provide the opportunity to investigate the effects of individual rye chromosomes following the formation of addition lines.

A parallel investigation was done for each set of addition lines, their corresponding octoploid triticale parent, as well as their wheat and rye progenitors, using AFLP analyses. The results indicated that the AFLP profile of each addition line was very similar to its corresponding triticale parent (Fig. 1). About 90% (data not shown) of the lost rye bands in triticales were observed as missing in the addition lines, and almost 100% of the missing rye bands in the addition lines were also absent in the corresponding triticales. Since there was a high similarity of band loss between a triticale and its corresponding addition lines, the current data did not reveal any different effects caused by different rye chromosomes. This was also because that some of the lost rye bands in triticale were present in two or more addition lines. The overall results suggested that genome alteration observed in wheat–rye addition lines was similar to the genome alteration that occurred in triticales. Since the genome constitutions of the addition lines were much simpler than the corresponding triticale(s), the data implied that addition lines are alternative materials to triticale for use in evolutionary studies of polyploids.

Discussion

This research demonstrated that genomic sequence changes, primarily sequence elimination, were very common in triticale and that those events predominantly and consistently targeted the rye genome regardless of the materials used. The results were in agreement with the findings from wheat (Ozkan et al. 2002; Feldman and Levy 2003) and *Brassica* (Song et al. 1995), where sequence loss was observed as an immediate response to allopolyploidization. However, in wheat, the lost fragments represented specific sequences (Feldman et al. 1997; Liu et al. 1998*a*, 1998*b*; Ozkan et al. 2001), which could be derived from either of the parents, rather than selectively targeting one of the parental genomes. In addition, the degree of variation was much higher in triticale than any of the other polyploids studied. This could be due to the fact that the parental relationship of triticale is intergeneric (i.e., *Triticum* × *Secale*) rather than interspecific. The wheat cytoplasmic background may be another

Table 4. Numbers of RFLP fragments of each banding pattern and the percentages of presence or absence of parental bands.

			CS×I		H×K		C×S		C×U		Total	
W	R	T	No.	%	No.	%	No.	%	No.	%	No.	%
+	–	+	496	97.8	556	98.8	428	94.5	403	97.6	1883	97.3
		–	11	2.2	7	1.2	25	5.5	10	2.4	53	2.7
–	+	+	99	35.2	85	39.2	107	37.8	104	42.1	395	38.4
		–	182	64.8	132	60.8	176	62.2	143	57.9	633	61.6
+	+	+	56	98.2	32	97	47	95.9	62	98.4	197	97.5
		–	1	1.8	1	3	2	4.1	1	1.6	5	2.5
–	–	+	54		62		74		60		250	
Total			899		875		859		783		3416	

Note: CS×I, 'Chinese Spring' × 'Imperial'; H×K, 'Holdfast' × 'King II'; C×S, 'Cocorit 71' × 'Snoopy'; C×U, 'Cocorit 71' × 'UC90'; W, wheat; R, rye; T, triticale; +, present; –, absent.

factor in determining the direction and amount of sequence elimination or changes. Song et al. (1995) reported different RFLP patterns within one pair of reciprocal interspecific crosses of *Brassica*, indicating cytoplasm had a potential role in the directional changes of polyploid genomes. However, the effect of cytoplasm in triticale could not be analyzed because a rye × wheat hybrid (i.e., when rye is used as maternal parent) is extremely rare and unstable.

The results also showed that the degree of rye genome variation was similar in all the triticales studied, regardless of the ploidy levels or AFLP primers used, but that the wheat genome conservation in octoploid triticales was much higher than that in hexaploid triticales. The data were in agreement with the study by Boyko et al. (1984) who found that DNA content was decreased about 9% in octoploid triticale and decreased up to about 28%–30% in hexaploid triticale. It is not clear how the genomes from wheat and rye interact differently under different triticale ploidy levels. The higher percentage of wheat genome dosage in octoploid triticale when compared with hexaploid triticale is certainly a key factor associated with this phenomenon.

The results also showed that when a parental AFLP band was present in both progenitors, it tended to be conserved in the triticale more easily than if it was present in just one of the progenitors. This suggested that genome alteration in triticale was likely to occur more frequently within regions of sequences with less similarity compared with highly syntenic regions containing common progenitor sequences.

The comparison of E–M and P–M primers indicated that repetitive sequences in triticale were involved to a much higher degree of sequence elimination or variation as compared with low-copy sequences. The vast magnitude of variation within repetitive sequences could provide an underlying mechanism to stabilize the triticale genome. In polyploid wheat, sequence elimination was speculated to provide a physical basis for diploid-like meiotic behavior during allopolyploidization (Ozkan et al. 2002; Feldman and Levy 2003). Similarly, genomic sequence elimination or variation was probably a driving force to promote the cytological process during and after triticale formation. The variation revealed by P–M primers and cDNA-probed RFLP analyses indicated that low-copy sequences were also involved in the cytological diploidization process of polyploid formation, but more importantly, that variation probably facilitated the evolutionary process of genetic diploidization, in which the process tended to change or silence redundant genes (Ma and Gustafson 2004). Polyploidization-induced gene silencing events have been reported in *Arabidopsis* (Comai et al. 2000; Lee and Chen 2001; Madlung et al. 2002), cotton (Adams et al. 2003), and wheat (Kashkush et al. 2002, 2003). The present RFLP data indicated that 61.6% of the rye expressed sequences were changed, while only 2.7% of the wheat expressed sequences changed (Table 4), suggesting that a large number of genes from rye were selectively inactivated because of sequence loss or modification.

In addition, both AFLP and RFLP analyses showed new bands that were not present in either of the parents. These new fragments might be derived from altered parental bands arising from genome modifications like cytosine methylation or structure variation after the rye genome had been introduced into wheat. Considering that some of the genome variation did not cause the appearance of new bands since a new band could be hidden by a parental band if they happened to have the same molecular size, the real percentage of overall genome variation could be much higher than detected (Ma et al. 2002).

The present data showed that some of the genome variation might be associated with concerted evolution. Concerted evolution has been reported in repetitive sequences or gene families. In cotton, rDNA arrays are homogeneous, or nearly so, in all diploids and tetraploids examined (Wendel et al. 1995). Because these arrays occur at four chromosomal loci in allopolyploid cotton, the authors concluded that repeats from the different diploid progenitors must have become homogenized by an interlocus concerted evolution. Furthermore, phylogenetic analysis demonstrated that interlocus concerted evolution operated in both directions, i.e., of the five tetraploid (AADD genome) cotton species studied, rDNA from four of them had been homogenized to a D-genome repeat type, whereas sequences from one tetraploid species had concerted to an A-genome type (Wendel et al. 1995). Similar results were also documented from other polyploid plants, including *Microseris* (van Houten et al. 1993; Roelofs et al. 1997), *Saxifraga* (Brochmann et al. 1996) and *Paeonia* (Sang et al. 1995; Zhang and Sang 1999). The current study demonstrated that low-copy genes could also be-

come homogenized by an interlocus concerted evolution as exemplified in Fig. 2 where the rye parental band was homogenized to a wheat-like band.

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